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Improving the discovery of secondary metabolite natural products using ion mobility-mass spectrometry

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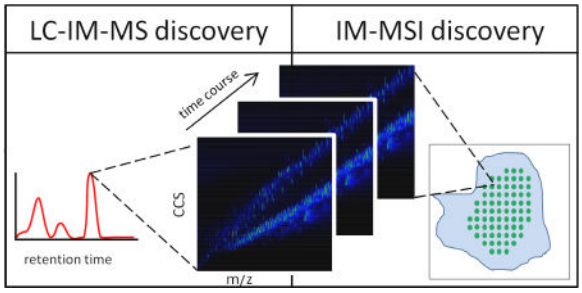
Abstract

Secondary metabolite discovery requires an unbiased, comprehensive workflow to detect unknown unknowns for which little to no molecular knowledge exists. Untargeted mass spectrometry-based metabolomics is a powerful platform, particularly when coupled with ion mobility for high-throughput gas-phase separations to increase peak capacity and obtain gas-phase structural information. Ion mobility data are described by the amount of time an ion spends in the drift cell, which is directly related to an ion's collision cross section (CCS). The CCS parameter describes the size, shape, and charge of a molecule and can be used to characterize unknown metabolomic species. Here, we describe current and emerging applications of ion mobility-mass spectrometry for prioritization, discovery and structure elucidation, and spatial/temporal characterization.

Graphical abstract



Keywords

Ion mobility; Mass spectrometry; Ion mobility-mass spectrometry; Secondary metabolite; Natural product

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Introduction

Advances in microbial genome sequencing have revealed cryptic secondary metabolite gene clusters that are thought to be unexploited sources of new bioactive compounds for discovery [1,2]. Expression of secondary metabolites can be an adaptive response, thus screening an organism under a variety of environmental stimuli to induce silent biosynthetic gene clusters could expand the catalog of natural products (NP) [3]. The analysis of complex chemically-diverse samples and identification of NP candidates is challenging. Untargeted metabolomics, which aims to comprehensively measure all analytes within a biological sample, has the potential to address this chemical diversity. A mass spectrometry (MS)-based metabolomic platform is a powerful approach with high sensitivity and specificity [4]. In a typical experiment, the MS scans over a defined mass range to detect ions related to metabolites of interest. The number of compounds detected depends on the resolving power of the separation platform(s). Pre-ionization separations are routinely interfaced with MS; liquid chromatography (LC) is widely selected [5,6] since a majority of NP are non-volatile. Coupling LC with MS increases peak capacity, although there is a concomitant decrease in throughput. Ion mobility (IM) is a high-throughput separation technique that allows for rapid (μs to ms) separation of molecules [7] and has shown utility for studying a variety of natural products sources [8–10].

Ion mobility is a form of gas-phase electrophoresis that is readily coupled to mass spectrometry. In temporally dispersive IM methods, ions traverse an electric field colliding with a carrier buffer gas. The number of collisions encountered depends upon an ion's physical properties (charge state, size, and shape), which are described by a collision cross section (CCS). Ions with a low mass and compact structure arrive at the mass analyzer with short drift times, while larger and bulkier molecules elute from the drift cell later. Ultimately, the ion mobility dimension increases the signal-to-noise ratio (S/N) in MS measurements. This report describes emerging applications for IM-MS analyses including feature prioritization strategies for downstream natural product discovery and structural elucidation, as well as ion mobility-MS imaging (MSI) for secondary metabolite spatial characterization. For a much more detailed survey of ion mobility-mass spectrometry, the reader is referred to several comprehensive reviews describing IM-MS theory, instrumentation, and analyses [7,11–13].

IM-MS for prioritization

IM is an appealing technology for NP prioritization efforts owing to its ability to separate molecules based on their gas-phase structural conformation. Unlike primary metabolites which are largely composed of conserved functional groups, secondary metabolite structures have diverse physicochemical properties (e.g., variable molecular weight, atom type, cyclization, degree of oxidation, etc.) [14]. This diversity can affect structural conformation and result in discrete regions of conformational space along trendlines being occupied by various molecular classes in mobility-mass correlations [15,16], as shown in (Figure 1a). Deviations of species from predicted trendlines can be exploited for secondary metabolite prioritization. Recent work has shown that a peptide natural product from cave actinomycetes [17] and halogenated natural products (i.e., particularly attractive lead

compounds [18]) derived from crude cyanobacteria extracts [9] deviate from predicted or experimentally-derived mobility trendlines.

Prioritization efforts can also leverage IM's ability of IM to enhance analytical figures of merit, particularly when combined with LC [19]••[20], although a variety of other front-end separation techniques have been coupled with IM-MS platforms [21]. Basit and coworkers recently showed the ability for an untargeted LC-IM-MS-based lipidomics workflow to detect changes in mouse brain tissue. Specifically, the ion mobility separation dimension uncovered a subclass of dysregulated phospholipids that were not initially revealed by conventional prioritization strategies (i.e., principal component analyses)[22]. Other LC-IM-MS studies have shown the ability to prioritize features via self-organizing maps (SOM) analyses of response trends to either multiplexed environmental stimuli [23] or co-culture with challenger organisms [24] •• designed to elicit potential induction of cryptic or silent gene clusters.

IM-MS for experimental flexibility

Numerous IM-MS instrument designs have been utilized in omics discovery research, and data showcase the benefit of incorporating an IM separation at a variable position within an instrument's configuration or experimental method. Performing IM prior to MS analysis enables ion signal filtering, for example, using Differential Mobility Spectrometry (DMS) to suppress chemical noise and separate small isobaric molecules [25], and Field Asymmetric waveform Ion Mobility (FAIMS)-MS to detect lower abundance lipid classes by separating a highly abundant protein species [26]•.

Many metabolomics approaches utilize high energy MS fragmentations to generate tandem spectra for identifications. These experiments focus on identifying features of interest by matching MS/MS spectra with reference spectra for candidate annotations. Spectrum matching can be particularly challenging for data independent acquisitions where fragmentation for many ions occurs simultaneously resulting in combined MS/MS spectra. The inclusion of IM prior to fragmentation facilitates deconvolution of MS/MS spectra through correlation of drift time (DT, from the mobility dimension) and retention time (RT, from the LC dimension) with precursor and product ions. This approach has been successfully used for the discovery of novel secondary metabolites and subsequent structure elucidation in mutant *Nocardiopsis* strains [27]. Mobility-separated spectra are also particularly advantageous for distinguishing isobars in complex samples [19]•[28]•. Recent experiments have shown the utility of an IM dimension for more accurate quantification of individual isobaric species and deconvolution of their combined MS/MS spectra [29].

Performing fragmentation prior to IM analysis also has demonstrated utility. Mobility-separated product ion data was used to guide neutral- and phospholipid identifications [30]•[31], a particularly challenging effort due to the existence of common structural moieties and large number of lipid isomers. Finally, an IM-MS instrument can be configured to enable fragmentation prior to *and* after mobility separation. This approach aided localization of fatty acyl and double bond positions in phosphatidylcholines [32]. An extension of this configuration incorporates in-source fragmentation to generate MS^4 data

and has enabled the identification of co-eluting isobaric polycyclic polyprenylated acylphloroglucinols, bioactive components of *Garcinia* plants, not previously resolved by conventional LC-MS based methods [33]•.

IM–MS for dereplication, identification, and structural elucidation

The process of dereplication (to discriminate novel versus previously elucidated natural products) is crucial in discovery-based MS workflows [34]. Methods to facilitate dereplication efforts via crowd-sourcing analyses have been employed (such as those available through the Global Natural Product Social Molecular Networking (GNPS) website [35]), though many features remain unknown unknowns (i.e., unidentified novel molecules). Analytical advances leading to increased molecular information content should expedite dereplication and elucidation efforts. In omics-based LC-MS/MS experiments, species are characterized with a multi-coordinate set of descriptors (e.g., retention time, accurate mass, isotopic distribution [36,37], MS/MS pattern [38]) to generate candidate identifications. The opportunity to include the IM dimension (i.e., CCS) as a complementary robust measurement to increase specificity and molecular information content is recognized [13,39,40]. Experimental CCSs can be derived directly from drift time measurements [via Drift Tube Ion Mobility Spectrometry (DT IMS)] or by performing a non-linear calibration with an appropriate calibrant under defined conditions [41]•• [via Traveling Wave Ion Mobility Spectrometry (TW IMS)]; inter-lab reproducibility has been established [39].

Though experimental CCS values are valuable physicochemical descriptors, their utility is currently the greatest for known unknown compounds (i.e., known compounds pending identification). Multiple known unknown candidates may meet the accurate mass measurement and isotopic abundance pattern criteria, thus filtering with published CCS values has the potential to reduce the number of candidates to support an identification (Figure 1b). Previously established CCS values are often unavailable. When a compound is labeled as an unknown unknown, CCS alone is not a discriminating identifier. In these cases, experimental IM data can also be used in conjunction with computational methods that simulate 3-D structural conformations and calculate theoretical CCS values [42] [43]• to guide identification of unknown unknowns; predicted conformations consistent with experimental data are prioritized for evaluation (Figure 1c). Recent works incorporating molecular dynamic simulations have allowed for the characterization of a dipeptide-amyloid β complex [44]• and drug metabolite structural isomers [45]•.

IM-MSI for spatial characterization

Multi-dimensional IM-MS metabolomics data allow for separations of ions based on their structural and physicochemical attributes, including hydrophobicity, mass, and collision cross section along with fragmentation data (Figure 1b). Mass spectrometry imaging (MSI) generates data with yet another level of knowledge, positional context, to interrogate spatial distributions of molecules and has been successfully combined with IM separations for added sensitivity. The ionization source of MSI instruments must be amenable to sampling the surface of a biological substrate (e.g., tissue, agar, etc.) and coincide with experimental goals (sample preparation, image resolution, etc.) [46]. Matrix Assisted Laser Desorption

Ionization (MALDI), Desorption Electrospray Ionization (DESI), and Liquid-Microjunction-Surface Sampling Probe (LMJ-SSP) have demonstrated success for IM-MSI applications [46–51] [52]•. After matrix application on the surface, MALDI imaging analyses are typically performed *in-vacuo* with relatively high spatial resolution (dictated by the size and shape of the laser); ambient ionization techniques, such as DESI and LMJ-SSP, require minimal to no sample preparation allowing samples to be analyzed in native state, but have more moderate spatial resolution [53]. Fernandez and coworkers used an IM separation in an effort to compensate for the limited spatial resolution of DESI [50]. The incorporation of DMS as a front end ion filter enabled increased sensitivity by improving S/N, effectively rendering higher quality images. A recent study used an interchangeable source to compare DESI and MALDI ionization of lipids in brain tissue on a single TWIM-MS instrument [52]•. The DESI IM configuration was particularly advantageous for this work as a trendline comprised of fragile lipid species was revealed, though several species with lower DESI ionization efficiencies were ionized better by MALDI, rendering complementary data.

Inherently, MSI does not enable chromatographic separation prior to imaging. Identification of ions detected in an MSI experiment is therefore challenging, owing to the m/z interferences (isobaric compounds and in-source fragmentation) [54] that confound the spatial data interpretation (Figure 1d). Further, the lack of an LC RT descriptor results in the use of accurate mass and isotopic abundance pattern alone for identification efforts. The ability of IM-MSI to address these limitations has been explored. Applications have shown the ability to reduce background chemical noise and increase sensitive and specificity, for example, to resolve poly-sialylated gangliosides in murine brain sections [52] and enable high-throughput localization and identification of recombinant biocatalysts in live bacterial colonies [55]•. Advances in IM-MSI technology will continue to benefit discovery-based research, particularly for 3-D imaging approaches to capture spatial profiles of molecular interactions [34] and investigations of native environments for biological systems exhibiting steep surface topologies [56].

Concluding remarks

IM-MS analyses can facilitate biological discovery research by providing an additional dimension of sensitive and specific physicochemical data for metabolite prioritization. LC-IM-MS measurements are particularly advantageous for natural product discovery where multiple descriptors (see Fig 1b.) are used to minimize dereplication efforts. Structural elucidation is a more arduous task. While IM will certainly augment identification confidence, pre-ionization separations/chromatography and MS/MS are still necessary for MS-based elucidation.

The full potential for massive data sets generated using IM-MS-based spatial and/or temporal experiments is tantalizing, though hurdles related to the mining the data must be overcome. New software tools need to be developed to extract and process high-dimensionality data in order for researchers to be able to interpret and manage findings. Additionally, data storage for copious large datasets is a legitimate concern. The IM field is currently determining the boundaries with which we can utilize CCS measurements for discovery and structural elucidations. Innovations in IM technology, specifically leading to

high mobility resolution analyses [57–60], MSI technology, and automated data analysis workflows incorporating both theoretical and experimental CCS data, should have far reaching implications in the natural product, and thus human health, research communities. Though we focused many examples on natural product-based secondary metabolite research, the utility of IM is applicable for primary and secondary metabolites in any biological system.

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•• of outstanding interest

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Highlights

- IM techniques separate ions in the gas-phase based on size, shape, and charge
- Deviations from mobility-mass trend lines can be prioritized for identification
- The inclusion of IM enables flexible MS experimental designs
- Collision cross section values can be used as physicochemical descriptors
- IM enhances imaging MS with post-ionization separation of isobaric species

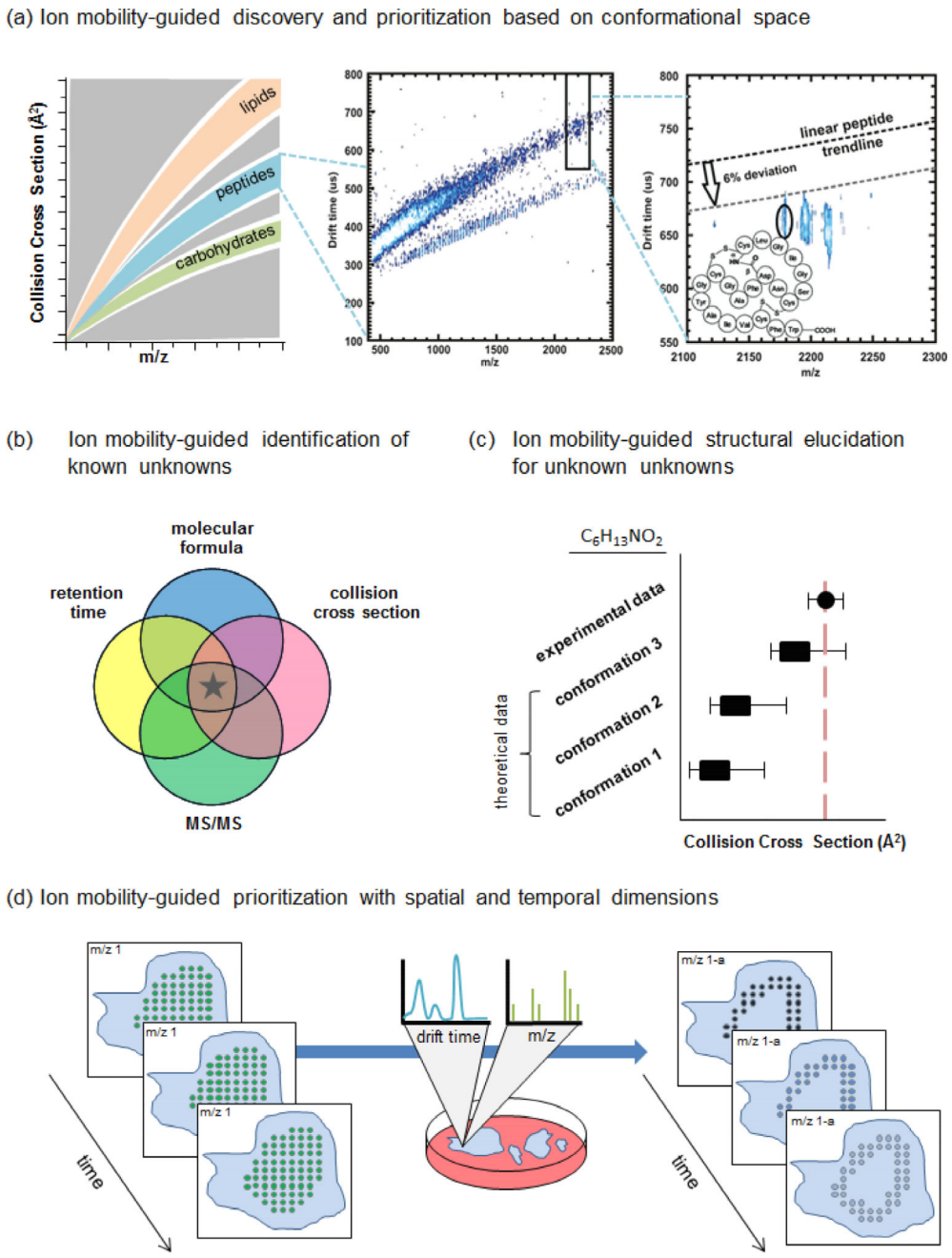

Figure 1.

Applications of metabolomic workflows utilizing ion mobility-mass spectrometry for discovery of secondary metabolite natural products. (a) (Left) IM-MS mobility-mass correlation plots reveal trends of various molecular classes within conformational space. (Middle) Deviations of species from predicted trendlines can be exploited for secondary metabolite prioritization. (Right) This region annotates a peak with dense gas-phase packing efficiency that falls nearly 12% below the linear peptide trendline for this m/z. This compound was identified as siamycin II, a tricyclic peptide, and confirmed by isolation and NMR analyses. Reproduced with permission from [17]. Copyright 2012 American Chemical

Society. (b) The Venn diagram represents candidate identifications based on individual data dimensions. Mass spectrometry yields accurate mass and isotope pattern information that can be used to determine molecular formula, liquid chromatography yields retention time, and ion mobility yields drift time/CCS. The goal of including multiple descriptors is to filter candidate space and remove false positives, ultimately leading to a true positive identification (represented here by a star). (c) Computational methods can be used to simulate 3-D structural conformations and calculate theoretical CCS values for comparison with experimental data. Conformations that are consistent with the experimental CCS range are prioritized for structural elucidation. In this mock example, three conformations were predicted for an unknown species with a molecular formula of $C_6H_{13}NO_2$. Their theoretical CCS values were compared with the experimental CCS value, revealing conformation 3 as a plausible structure. (d) MSI experiments generate m/z spectra for each spatial coordinate. The integration of an ion mobility dimension enables isobaric species to be separated resulting in more accurate ion images. In this example, m/z 1 appears with uniform signal (green dots) across a sample. Mobility data revealed an isobar, m/z 1-a, with signal (blue dots) that appears as a border around the sample. Further temporal data would enable tracking of this spatial data as a function of time.